Structural and Functional Disruption of Vascular Smooth Muscle Cells in a Transgenic Mouse Model of Amyloid Angiopathy

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The deposition of amyloid A β peptide in the wall of cerebral vessels (cerebral amyloid angiopathy), can lead to weakness and rupture of the vessel wall, resulting in hemorrhagic stroke. The Tg2576 transgenic mouse line, overexpressing mutant amyloid precursor protein in an age-dependent manner, forms amyloid angiopathy morphologically similar to that seen in the human. We report here the structural and functional disruption of smooth muscle cells (SMCs) in the walls of pial vessels affected by amyloid deposition in the Tg2576 mouse. We demonstrate, using multiphoton imaging, that the arrangement of SMCs becomes disorganized before the onset of cell death, and that these disorganized SMCs are unable to respond appropriately to application of endothelial-dependent and endothelial-independent vasodilators in a closed-cranial window preparation. (*Am J Pathol* 2001, 158:1065–1071)

Cerebral amyloid angiopathy (CAA), one of the leading causes of stroke because of lobar hemorrhage in the elderly,¹ results from deposition of A β peptide in the wall of cortical and leptomeningeal vessels. CAA frequently co-occurs with Alzheimer's disease (AD), and the A β peptide that accumulates around vessels in CAA also accumulates in plaques in AD.^{2,3} Neuropathological studies show that A β deposition is associated with structural changes in the vessels: localized loss of smooth muscle cells (SMCs), fibrinoid necrosis, weakening of the vessel wall, and in some cases, rupture and hemorrhage into the brain.³ It is unknown whether the function of the vessels is affected by amyloid deposition, because the caliber of the vessels is too small to be visualized by clinical imaging techniques.

Mice transgenic for the amyloid precursor protein (APP), originally developed as a model of AD senile plaque formation, also serve as an excellent model of CAA.^{4–5} Tg2576 mice express human APP carrying the Swedish mutation (HuAPP695.K670N-M671L), a double mutation in the sequence of APP that favors the formation

of the 40-amino acid A β peptide.⁶ Amyloid deposition starts at 10 to 12 months of age⁴ in both the neuropil, as senile plaques, and in cerebral vessels, as CAA. This vessel-associated deposition of amyloid, like that in the parenchyma, seems to be affected by the presence of ApoE in the extracellular environment.⁷

The present work tests the hypothesis that vessel-associated amyloid in these animals interferes with the anatomical integrity and physiological responses of affected vessels. We report, using double-labeling of SMCs and vessel-associated amyloid and three-dimensional reconstruction of the vessel wall with multiphoton laser-scanning microscopy, an age-dependent disruption of SMCs in the wall of affected leptomeningeal vessels, initially without SMC loss. We then demonstrate that this disruption of SMC organization interferes with the ability of the vessel to respond to both an endothelial-dependent vasodilator (ACh) and an endothelial-independent vasodilator [sodium nitroprusside (SNP)], suggesting an underlying loss of coordinated SMC function in affected vessels.

Materials and Methods

Transgenic Mice

Tg2576 mice expressing hAPP(Sw) under the hamster prion protein promoter were obtained from a colony started with a breeding pair from K. Hsiao (University of Minnesota). These animals have been shown to develop age-dependent amyloid angiopathy as well as cortical and hippocampal amyloid plaques similar to those seen in AD.⁴ Eight animals carrying the transgene and eight nontransgenic littermates were used for the anatomical measurement of SMCs in pial vessels. Mice of each genotype were of two age groups, a young group at 6 months of age, and an older group at 14 months of age (Table 1). These same groups of animals were used for the vessel reactivity experiment. Additionally, amyloid deposition and SMCs were imaged in an older group of

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Table 1. Baseline Physiology of TG2576 Mice

Group	Age (months)	n	pH	Blood CO ₂ (mmHg)	Blood pO ₂ (mmHg)	Mean BP pre (mmHg)	Mean BP post (mmHg)	Vessel diameter (μm)
6 month Tg–	5.3 ± 0.6	3	7.41 ± 0.01	30.6 ± 3.4	169.2 ± 57.8	95.0 ± 5.6	91.7 ± 4.7	25.7 ± 2.3
6 month Tg+	6.3 ± 0.6	3	7.32 ± 0.05	37.5 ± 6.0	127.6 ± 20.7	72.0 ± 13.0	64.7 ± 18.0	28.3 ± 0.6
14 month Tg–	13.7 ± 1.2	3	7.39 ± 0.06	33.0 ± 4.5	166.6 ± 23.0	66.0 ± 11.0	62.7 ± 12.7	31.3 ± 6.4
14 month Tg+	14.0 ± 2.6	5	7.33 ± 0.09	33.4 ± 3.2	149.0 ± 22.7	65.0 ± 7.6	61.3 ± 3.2	28.3 ± 2.9

three transgene-positive animals, aged 24.7 ± 2.3 months.

SMC Imaging

After *in vivo* vascular reactivity measurements (see below), animals were sacrificed by an overdose of anesthetic (halothane). Intact crania were removed and fixed in paraformaldehyde [4% in Tris-buffered saline (TBS)] for several days. The presence of the intact skull and craniotomy identified the same population of vessels whose dilation was measured *in vivo*. The brain was then removed, washed with TBS, treated with 0.5% Triton-X in TBS for 20 minutes, washed again, then incubated in 1% bovine serum albumin in TBS for 20 minutes to minimize nonspecific background staining. Vessels were stained with a combination of Alexa-568 phalloidin (50 μl stock solution/2 ml; Molecular Probes, Eugene, OR) and thioflavine S (thioS) (0.005%; Sigma Chemical Co., St. Louis, MO) in 1% bovine serum albumin in TBS. After 20 minutes in the staining solution, the brains were washed with TBS, and stabilized within a plastic dish with molten bone wax. The brains were covered in TBS, into which a dipping microscope objective was lowered, for imaging.

A BioRad 1024MP multiphoton imaging system (BioRad, Hercules, CA) with a Ti:Sapphire laser (Spectra Physics) operating at 750 nm, with an output power of 25 mW at the back aperture of the objective, was used for imaging. The system was mounted on an upright Olympus BX-50 microscope, equipped with long working distance dipping objectives ($\times 10$, NA 0.5; $\times 60$, NA 0.8). Custom designed external detectors (W. Zipfel, Cornell University) were used to enhance detection of emitted light. The filter set used separated emitted light into three channels: 360 to 430 nm, 485 to 505 nm, and 525 to 650 nm. The thioS and Alexa-568 signals fell clearly into the first two and third channels, respectively.

SMC Density Measurement

Series of $\times 60$ optical sections spaced 2 μm apart were taken through branches of the anterior and middle cerebral arteries on the dorsal aspect of the intact brain. Each optical section was acquired at slow scan speed, with Kalman filtering of two successive scans for noise reduction. Vessel structure was then reconstructed by a maximum intensity projection of the stack of optical sections. A computer-generated index line of random length was drawn perpendicular to the vessel diameter (Scion Image). Linear SMC density was calculated as the number

of SMCs along this line divided by the length of the line in μm. Three vessels were measured from each animal; to minimize distortion of the measured SMC density by a sloping vessel, the selected vessels were those whose longitudinal axis most nearly matched the imaging plane. Although the obvious presence of amyloid on the vessels made blind selection with respect to genotype impossible, the measurement of phalloidin-stained SMCs was blind with respect to the age of the animal imaged.

Animal Preparation for Vascular Reactivity Measurement

All experiments were conducted in accordance with National Institutes of Health and Massachusetts General Hospital Institutional guidelines. Animals were allowed food and water *ad libitum*. Anesthesia was induced with 2.5% halothane and maintained in 1.0% halothane in 67% N₂O and 33% O₂. Mice were intubated transorally, placed in a stereotaxic frame, and ventilated artificially (SAR-830/P; CWE, Ardmore, PA). End-tidal CO₂ was continuously monitored by a microcapnometer (Columbus Instruments, Columbus, OH). The femoral artery and vein were cannulated with a polyethylene catheter (PE-10, Intramedic; Becton Dickinson, Mountain View, CA) for continuous arterial blood pressure and heart rate monitoring and for drug infusion. α-chloralose (80 mg/kg i.v.) was injected and halothane was withdrawn gradually for deepening of anesthesia. Supplemental doses of α-chloralose were given as needed to maintain a stable level of anesthesia, which was periodically tested by arterial blood pressure and heart rate response to tail pinch. Arterial blood gas and pH were analyzed before drug superfusion. Rectal temperature was maintained at 37°C with a thermostatically controlled mat (temperature control; FHC, Brunswick, ME).

Closed Cranial Window Preparation

Techniques used for measurement of vessel diameter changes in mice were similar to those described. The head was fixed in a stereotaxic frame, and the skull exposed by a longitudinal skin incision. A stainless steel cranial window ring (7.0-mm inner diameter, 1.7 mm in height) containing three flow ports was adhered to the skull in a loop of bone wax. A craniotomy (2 × 1.5 mm) was made in the left parietal bone within the ring of the window. The dura was then opened while the brain surface was superfused with artificial cerebrospinal fluid

(ACSF). A cover glass was placed on the ring and affixed with dental acrylic. The ports were attached to inflow and outflow connections, allowing for superfusion of solutions directly onto the exposed brain; the volume under the window was ~ 0.1 ml. ACSF was as follows (in mmol/L): Na^+ , 156.5; K^+ , 2.95; Ca^{++} , 1.25; Mg^{++} , 0.67; Cl^- , 138.7; HCO_3^- , 24.6; dextrose, 3.7; and urea, 0.67. ACSF was kept at pH 7.35 to 7.45 by equilibration with 6.5% CO_2 , 10% O_2 , and 83.5% N_2 . ACSF was circulated by an infusion pump (0.4 ml/min) via PE-50 tubing connected to the inlet port. Intracranial pressure was maintained at 5 to 8 mmHg by adjusting the outlet tubing to an appropriate height above the level of the window; ACSF temperature within the window was maintained at 36.5 to 37.0°C.

Vessel Diameter Measurement

Pial vessels were visualized with a video microscope system comprised of an intravital microscope (Leitz, Germany), CCD video camera (model 1300; Cohu Inc., San Diego, CA), a camera controller (C2400; Hamamatsu Photonics, Hamamatsu, Japan), video monitor (Sony), and a video recorder (Panasonic). The images were continuously recorded on videotape. The diameter of a single pial arteriole (20 to 30 μm) was measured per experiment by a video width analyzer (C3161, Hamamatsu Photonics) and recorded using the MacLab data acquisition and analysis system. ACSF was superfused for 20 to 30 minutes until a stable baseline diameter was obtained. Acetylcholine (ACh) (10 and 25 $\mu\text{mol/L}$) and SNP (0.5 $\mu\text{mol/L}$; Sigma) dissolved in ACSF were then applied to assess vessel dilation. Drugs were superfused for 10 minutes, followed by ACSF superfusion for an additional 20 minutes for washout and return to baseline vessel diameter. The order of application of the two drugs was chosen at random. In a subset of animals ($n = 6$), two cumulative concentrations of ACh were superfused, without return to baseline between the low and high doses. For each application of drug, the maximum diameter change from baseline was compared. Vessel imaging and data analysis were performed without experimenter knowledge of the genotype or age of animal. Animals that exhibited significant hypotension ($n = 2$) or hypercapnia ($n = 1$) during the procedure were eliminated *a priori* from analysis.

Results

To assess the effect of this amyloid deposition on the structure of the vessel wall, SMCs were visualized in conjunction with amyloid using a combination of fluorescently tagged phalloidin and thioS. Phalloidin (phalloidin) binds to actin filaments, particularly F-actin, and has been used to visualize SMCs under a variety of conditions.^{8,9} Phalloidin stains vascular SMCs in fixed mouse pial vessels, and, conjugated to Alexa 568, can be imaged in a separate emission channel from thioS. In this way, the organization and number of SMCs in a length of pial arteriole can be studied relative to the surrounding

Figure 1. ThioS-positive amyloid angiopathy in the Tg2576 mouse. The intact fixed brain of a 16-month-old Tg2576 mouse was stained with thioS (0.005%) and imaged using two-photon excitation at 750 nm. This image is a montage of 4×8 z-series collected with a $\times 20$ objective. The midline of the brain is at the **top** of the figure, and the brain was oriented with the anterior pole to the **left**. Extreme curvature at the lateral edge of the brain interfered with montage generation, distorting the lowermost portion of the image. The middle cerebral artery emerges from behind the lateral edge of the brain on the **right**, and courses toward the midline. ThioS-positive vessel-associated amyloid, as well as superficial parenchymal thioS-positive plaques are clearly visible. Surface venules are seen as negatively stained background profiles. Scale bar (**upper right**), 600 μm .

amyloid. Staining could be accomplished in intact, fixed brains and three-dimensional imaging performed with multi-photon laser-scanning microscopy.

Figure 1 shows the pattern of amyloid angiopathy on leptomeningeal vessels of a 16-month-old Tg2576 mouse. This montage of 32 images illustrates how the involvement of the middle cerebral artery varies along its length, and is typical of all vessels examined. The larger caliber portion of the vessel seems to be the earliest and most severely affected, with the amyloid forming com-

Figure 2. Overexpression of mutant APP does not disrupt SMCs independent of amyloid deposition. Phalloidin-labeled SMCs in young (6 month) Tg2576 animals are arranged neatly around the circumference of the vessel, with no apparent space between adjacent cells. **a:** Phalloidin-stained SMCs in a pial vessel from a Tg⁻ animal. **b:** SMCs in a pial vessel of a Tg⁺ animal. Scale bar, 20 μ m.

plete rings around the circumference of the vessel. The classic segmental appearance of the amyloid is evident, and in the most severely affected portions of the vessel, the amyloid continues uninterrupted for stretches of several hundred μ m. Smaller size vessels have less amyloid, with sparser deposits, sometimes amounting to isolated slivers of amyloid on the vessel wall. Amyloid deposition appeared exclusively on the walls of arterioles, whereas venules, whose silhouettes appear in the figure background, remained unaffected.

The morphology of SMCs in amyloid angiopathy was studied at three ages. Nontransgenic littermates and transgenics too young for amyloid deposition (6 months) showed orderly arrangement of SMCs. SMCs were arranged circumferentially around the vessel, and packed adjacent to one another along the length of the vessel with no apparent space between them (Figure 2).

By contrast, thioS-positive amyloid substantially disrupts SMCs in 14-month and 24-month transgenic animals (Figure 3). Regions of sparse amyloid were characterized by shards of thioS-positive material between neighboring SMCs. More heavily affected portions of vessels showed amyloid encasing individual SMCs, distancing and eventually completely isolating them from neighboring cells. Although the organization of SMCs in affected vessels of the 14-month-old group was clearly

abnormal (Figure 3, a and b), SMCs seemed to have accommodated encroaching amyloid by contracting along their lateral dimension; SMC loss was not apparent along the length of the vessel. In accord with these qualitative observations, quantitative analysis (Figure 4) showed that there was no significant change in the linear density of SMCs within thioS-positive portions of vessels compared to thioS-negative portions of vessels in 14-month Tg2576 animals or compared to measurements in nontransgenic littermate controls.

The oldest group of animals studied (24 months), however, did lose SMCs along the length of the vessel in the areas of heaviest amyloid deposition (Figure 3, c and d). The density of SMCs along the length of the vessel was calculated for portions of vessels affected and unaffected by amyloid; by 24 months of age, amyloid-laden vessels lost over half the SMCs relative to unaffected vessels from the same animals. The SMC density in amyloid-free vessels or portions of vessels was not significantly different between the 14-month-old and 24-month-old age groups (Figure 4), nor did it differ significantly from SMC density in vessels from nontransgenic 14-month-old, nontransgenic 6-month-old, or transgenic 6-month-old animals. Subsequent examination of Nissl-stained histological sections revealed preservation of endothelial cells even in severely affected portions of the vessels. No evidence for hemorrhagic stroke was observed.

Loss of SMCs in the vessel wall, as seen in the 24-month-old animals, is certain to alter dilation in response to physiological or pharmacological stimulation; the consequence of disruption of SMCs, as seen in the 14-month-old animals, is unknown. We hypothesized that the presence of amyloid in the vessel wall would impair vessel function even before SMC loss. We therefore directly examined the physiology of pial vessels, using a closed cranial window preparation in the young (6 months) and older (14 months) transgenic and nontransgenic littermate control animals. We measured the change in vessel diameter to application of either acetylcholine (ACh), which causes endothelial-dependent vasodilatation through a nitric oxide-dependent mechanism,¹⁰ or SNP, a nitric oxide donor that acts directly on SMCs (Figure 4). To test the possibility that overexpression of the APP gene and overproduction of A β peptide has an effect on vessel function independent of amyloid deposition, we also measured vessel response in the 6-month-old transgenic and nontransgenic animals. The physiological parameters of the four measured groups are shown in Table 1. No significant differences were observed for arterial blood pH, CO₂, O₂, or baseline vessel diameter for the four groups ($P > 0.05$, analysis of variance). A significant difference was seen between the arterial blood pressure measurements for the young nontransgenic group and those for the other three groups ($P < 0.05$, analysis of variance), with the blood pressure both before and after vessel reactivity measurement being substantially higher in this younger group. No difference was seen, however, between the mean blood pressure before the experiment and the blood pressure after the experiment for any of the experimental groups.

Figure 3. Effect of amyloid deposition on SMCs in 14-month-old and 22-month-old Tg2567 animals. **a:** Phalloidin-labeled SMCs in the wall of a pial arteriole in a 14-month-old Tg2576 animal. **b:** ThioS-positive amyloid surrounding the vessels. SMCs are clearly disrupted in areas of amyloid deposition as compared to unaffected regions of the same vessel. SMCs surrounded by amyloid are disorganized and isolated, although there is no apparent loss of cells along the length of the vessel. **d:** SMC staining in a 24-month-old Tg2576 animal. **e:** ThioS-positive amyloid surrounding the vessel. At this age, overt loss of SMCs along the length of the vessel is evident, along with disruption of remaining cells. Regions of the vessel unaffected by amyloid, however, retain normal SMC organization. **c** and **f:** Superimposed color images showing both phalloidin (red) and thioS (green) staining. Scale bar, 20 μ m.

The percentage dilation to application of ACh and SNP for the four groups is shown in Figure 5. No difference in the response to either ACh or SNP was observed between the 6-month-old Tg+ and Tg- groups. By contrast, in the 14-month-old animals, vessel dilation was markedly attenuated in response to both doses of ACh and to SNP in four out of five transgenic animals as compared to the

nontransgenic group. Vessel dilation in these animals, in fact, was reduced to $\sim 25\%$ that of control animals. One outlier in the transgene-positive group showed essentially normal responses to both ACh and SNP, values that were six standard deviations away from the mean of the remaining members of the transgene-positive group. No difference in any of the physiological parameters of this individual animal could account for this remarkable discrepancy from other group members.

Figure 4. Quantitation of SMC density in amyloid-laden *versus* amyloid-free vessels in 14-month-old and 24-month-old Tg2576 mice. SMC linear density was measured as described. Density was measured in affected and unaffected vessels from both age groups. The 24-month-old amyloid-laden set of vessels has significantly smaller SMC density ($P < 0.01$, analysis of variance) than either the amyloid-free vessels from the same animal or amyloid-free vessels from younger transgenic and nontransgenic animals.

Figure 5. Response of pial vessels to ACh and SNP. Maximal percent dilation in response to ACh (10^{-6} mol/L) and SNP (0.5×10^{-6} mol/L) in 14-month-old Tg+ ($n = 4$ of 5, one outlier excluded) and Tg- ($n = 3$ of 3) mice. Bars are mean $\pm$ SD. *, $P < 0.05$ by analysis of variance.

After physiological study, the animals were perfused and prepared as noted above for detailed multiphoton laser-scanning microscopy analysis. On imaging, the vessels in the 14-month-old outlier were moderately involved with thioS-positive amyloid in the measured vessel segment, not dissimilar from other members of the group, although lacking the complete rings of amyloid present in the most severely affected vessels. Exclusion of data from this outlying animal results in highly significant ($P < 0.005$, analysis of variance) difference between the transgenic animals and nontransgenic littermates for both doses of ACh and for SNP. Inclusion of this outlier results in a more modest, but significant result for the 10^{-5} mol/L administration of ACh ($P < 0.05$), and nonsignificant differences for the higher dose of ACh and for SNP.

Discussion

Using Tg2576 transgenic mice, we examined the natural history of A β deposition in cerebral vessels and tested the hypothesis that amyloid deposition leads to both structural and functional disruption of affected vessels.

The data indicate that amyloid-associated disruption of SMCs impairs response to both endothelial-dependent and endothelial-independent vasodilators at an age pre-dating loss of SMCs in these vessels. Several possibilities for the mechanism of this interference of amyloid with vessel function exist. Amyloid may present a mechanical obstacle to vessel dilation, rendering the vessel wall relatively rigid. This possibility is supported by the long-standing observation that vessels with amyloid angiopathy fail to collapse in postmortem tissue, giving them the classic "stove-pipe" appearance.² The same inflexibility that maintains vessel diameter postmortem may physically restrict dilation *in vivo*. Alternatively, physical separation of adjacent SMCs by amyloid may disrupt contraction dependent on their coordinated action. A third possibility, given that the accumulation of amyloid around these vessels ultimately results in significant death of SMC, may be a low-grade toxicity of amyloid on the SMC that interferes with their ability to dilate appropriately, perhaps by altering expression of channel proteins (eg, Ca⁺⁺-dependent K⁺ channels¹¹) mediating nitric oxide-dependent relaxation in cerebral vessels.¹² Amyloid is toxic to endothelial cells in culture,¹³ and a mutant form of the A β peptide is toxic to SMCs in culture, although the 1-40 form that predominates in the Tg2576 mouse did not demonstrate direct toxicity to SMCs.^{14,15} The clear *in vivo* loss of SMC function, then, may reflect a preliminary stage in a cascade of events that leads to cell loss. The SMC loss seen in the Tg2576 mouse model of amyloid deposition parallels that previously described at the ultrastructural level in postmortem human AD cases.¹⁶ Intriguingly, such a model of amyloid-induced SMC dysfunction presents the possibility of restoring vessel function, if the amyloid can be cleared before SMC loss in affected vessels. The development of therapeutic approaches for amyloid clearance¹⁷ should enable the testing of this hypothesis in these animals.

Cerebral vessel function has been previously studied in mice overexpressing APP(Sw) on an FVB background (Tg1130H). These mice do not develop amyloid deposits, and die at a relatively young age. In contrast to the present results, the Tg1130H mice showed impaired endothelial-dependent, but not endothelial-independent, changes in cerebral blood flow.¹⁸ Differences in the age, background strain (the Tg2576 are on a C57 B/J1 F1 background), or the exact measurement protocols (blood flow *versus* vessel diameter) might also contribute to observed differences. Taken together, however, the data demonstrate profound impairment of the functional integrity of cerebrovascular responses because of overexpression of mutant APP and A β deposition, and imply that functional alterations are also likely to occur in CAA and AD. These *in vivo* results, together with observations that A β has a positive inotropic effect on aortic rings studied *ex vivo*,^{19,20} support the hypothesis that vessel-associated A β causes a physiologically relevant impairment of cerebrovascular vessel structure and function.

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